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Immunohistochemical Expression Analysis of the Human Interferon-Inducible Gene IFI16, a Member of the HIN200 Family, Not Restricted to Hematopoietic Cells

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ABSTRACT

This is the first description of an extensive immunohistochemical analysis of interferon (IFN)-inducible gene IFI16 expression in normal tissues. Immunohistochemical detection of IFI16 in paraffin-embedded tissues is achieved by using a polyclonal antibody raised against its C-terminal fragment that recognizes its three closely migrating isoforms in Western blotting. The results clearly indicate that IFI16 expression is not restricted to the hematopoietic compartment. In normal adult human tissues, it is prominent in stratified squamous epithelia and particularly intense in parabasal cells in the proliferating compartments, but it gradually decreases in the more differentiated suprabasal layers. Understanding of IFI16 expression *in vivo* is essential for interpretation of the results obtained from *in vitro* studies and elucidation of its physiologic role. The constitutive expression and wider distribution of IFI16 in normal human tissues, not restricted to the hematopoietic compartment, strongly support the possibility of an important role in cell differentiation that can be further modulated by other stimuli, such as IFN.

INTRODUCTION

INTERFERONS (IFNs) ARE CYTOKINES that modulate a variety of physiologic responses by exerting antiviral, immunomodulatory, and cell growth regulatory functions. They bind to cell surface receptors and activate the expression of many genes whose protein products directly or indirectly mediate the necessary biologic response.[1,2] There are hundreds of cellular genes that can be induced following IFN stimulation, but the molecular and biologic functions of many of these gene products often are not known. Among the proteins induced by IFNs are the members of the Ifi200 family in mice (Ifi202, Ifi204, Ifi203 and D3)[3–5] and their human counterparts (HIN200 family), including IFI16,[6] myeloid nuclear differentiation antigen (MNDA)[7] and absent in melanoma 2 (AIM2).[8] A 200-amino acid domain present singly or in duplicate is a structural motif found in all members.[9] Two domains, designated *a* and *b,* are contiguous in p202 and p204 but separated by a serine-threonine-proline (S/T/P)-rich spacer region in IFI16. The size of this spacer region is regulated by mRNA splicing. It contains one, two, or three copies of a highly conserved 56-amino acid S/T/P domain encoded by distinct exons.[10] The amino acid composition of the *a* and *b* domains in each member is highly conserved.

The 204 protein is encoded by the Ifi204 gene. It is a 72-kDa phosphoprotein that increases several-fold on treatment with IFNs, synthetic double-stranded RNA (dsRNA), lipopolysaccharide (LPS), or viral infection. The two 200-amino acid domains harbor an LXCXE motif that is a potential site for binding to the retinoblastoma (Rb) gene product.[11] Its overexpression inhibits cell proliferation in sensitive cell lines by delaying G_0/G_1 progression into S phase and impairing E2F-mediated transcriptional activity.[12,13] This growth arrest is accompanied by cell differentiation, as suggested *in vitro* by the finding that myoblasts overexpressing p204 mature into myotubes[14] and *in vivo* by the finding that monocytes over-

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expressing p204 mature into peritoneal macrophages.[15] Finally, our results have demonstrated that expression of a mutant p204 devoid of both Rb-binding motifs interferes with the mouse cytomegalovirus (MCMV) multiplication cycle. The Ifi204 gene is indeed transcriptionally activated by MCMV infection and is required for its replication.[16–18]

The human family has been designated hematopoietic IFN-inducible nuclear proteins (HIN200) because all the family members are expressed in hematopoietic cells, with some molecules showing a tightly regulated expression pattern in certain cell types. IFI16 is expressed in precursor $CD34^+$ stem cells and remains strongly expressed throughout lymphoid development and with monocyte precursors and peripheral blood monocytes.[19–21] However, it is not expressed in mature macrophages, nor is it found in cells of the erythroid and polymorphonuclear lineages. This expression pattern is similar to that of p204, which is constitutively expressed in mouse myelomonocytic cells.[15] Like p204, IFI16 binds retinoblastoma protein (pRb) both *in vitro* and *in vivo*. When overexpressed in human cell lines that do not normally express it, IFI16 retards cell growth by delaying progression from G_1 to S phase. Thus, it is now clear that both Ifi204 and IFI16 protein products modulate cell growth and perhaps *in vivo* differentiation. Some of their biochemical properties and molecular interactions have been delineated *in vitro,* whereas their physiologic role and systemic *in vivo* expression have not been determined. There are no published studies to date on the distribution of IFI16 *in vivo* outside the hematopoietic compartment. Full clarification of its expression would certainly serve to illustrate its physiologic role. In this study, rabbit polyclonal monospecific antibodies generated against the C-terminus fragment of IFI16 were used to investigate its tissue distribution in normal human sections by immunohistochemical analysis. We report a comprehensive immunohistochemical survey of IFI16 expression in normal human tissues. The results show that IFI16 is not restricted to the hematopoietic compartment but is highly expressed in the parabasal proliferating layer of the stratified epithelia, endothelial cells, and lymphocytes.

MATERIALS AND METHODS

Cells and treatment

The human cell lines U-937 (myelomonocytic leukemia) and HL-60 (promyelocytic leukemia) from the American Type Culture Collection (ATCC, Rockville, MD) were cultured in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (GIBCO IBRL, Gaithersburg, MD). HaCat cells were a gift from Dr. M. Tommasino (Deutsches Krebsforschungszentrum, Heidelberg, Germany) and were cultured in the same medium.

Recombinant human IFN-γ (rHuIFN-γ) (specific activity 4×10^{-7} U/mg protein) was used to treat cell cultures for 24 h before preparing total protein extracts.

Production and purification of antibodies against IFI16

Antibodies against IFI16 were produced by cloning the C-terminus IFI16 cDNA, that is, *Pvu*II-*Sma*I fragment from pBKS-IFI16 (kindly provided by J. Trapani, The Peter MacCallum Cancer Institute, Victoria, Australia), into the blunt-end *Sal*I unique site of pGEX-4T-2 vector (Pharmacia, Uppsala, Sweden) to create an in-frame fusion protein with the GST coding region. In-frame fusion was confirmed by DNA sequence analysis. The expression of C-terminus GST-IFI16 fusion protein in the *Escherichia coli* host AD202 was induced by treatment with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 3 h. The bacterial cells were harvested by centrifugation, resuspended in cold lysis buffer (0.5 mg/ml lysozyme, 25 mM Tris-HCl, pH 7.9, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 10% glycerol, 0.2% Triton X-100 containing 2 mM PMSF, 50 mM pepstatin A, and 50 mM leupeptin as protease inhibitors) and lysed by sonication. Fusion proteins were purified from the cleared lysate by glutathione-Sepharose affinity chromatography. Antisera against IFI16 were raised by injecting rabbits with the purified GST-IFI16 fusion proteins. The sera obtained af-

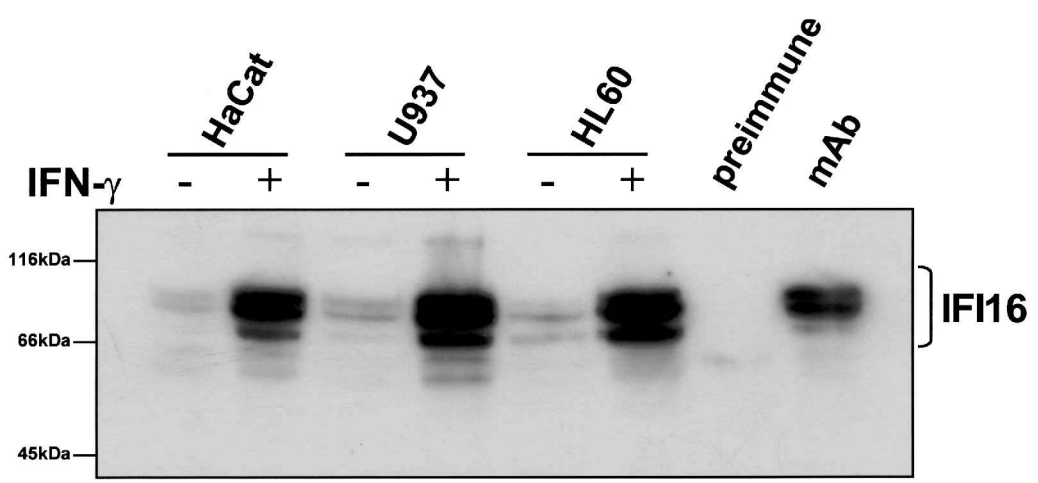

FIG. 1. Identification of IFI16 protein isoforms in IFN-treated cells. Whole cell lysates from HaCat, U-937, and HL-60, either untreated or treated with IFN-γ for 24 h, were run on 8.5% SDS-PAGE. Proteins were separated and probed with the affinity-purified rabbit polyclonal anti-IFI16 antibody. Anti-IFI16 mAbs were used as positive control (mAb), and preimmune serum was used as negative control (preimmune). Actin immunodetection was performed as an internal control. Molecular mass markers (kDa) are at left.

ter bleeding at 1 week after the fourth immunization were precipitated with ammonium sulfate at 45% saturation. The precipitate was then resuspended in phosphate-buffered saline (PBS) and purified on a protein A affinity column (Pharmacia) according to the specification of the supplier.

Immunoblotting

Cells were lysed in 3% SDS lysis buffer as previously described.[13] Insoluble material was removed by centrifugation. Protein concentration was determined by the Bio-Rad Dc Pro-

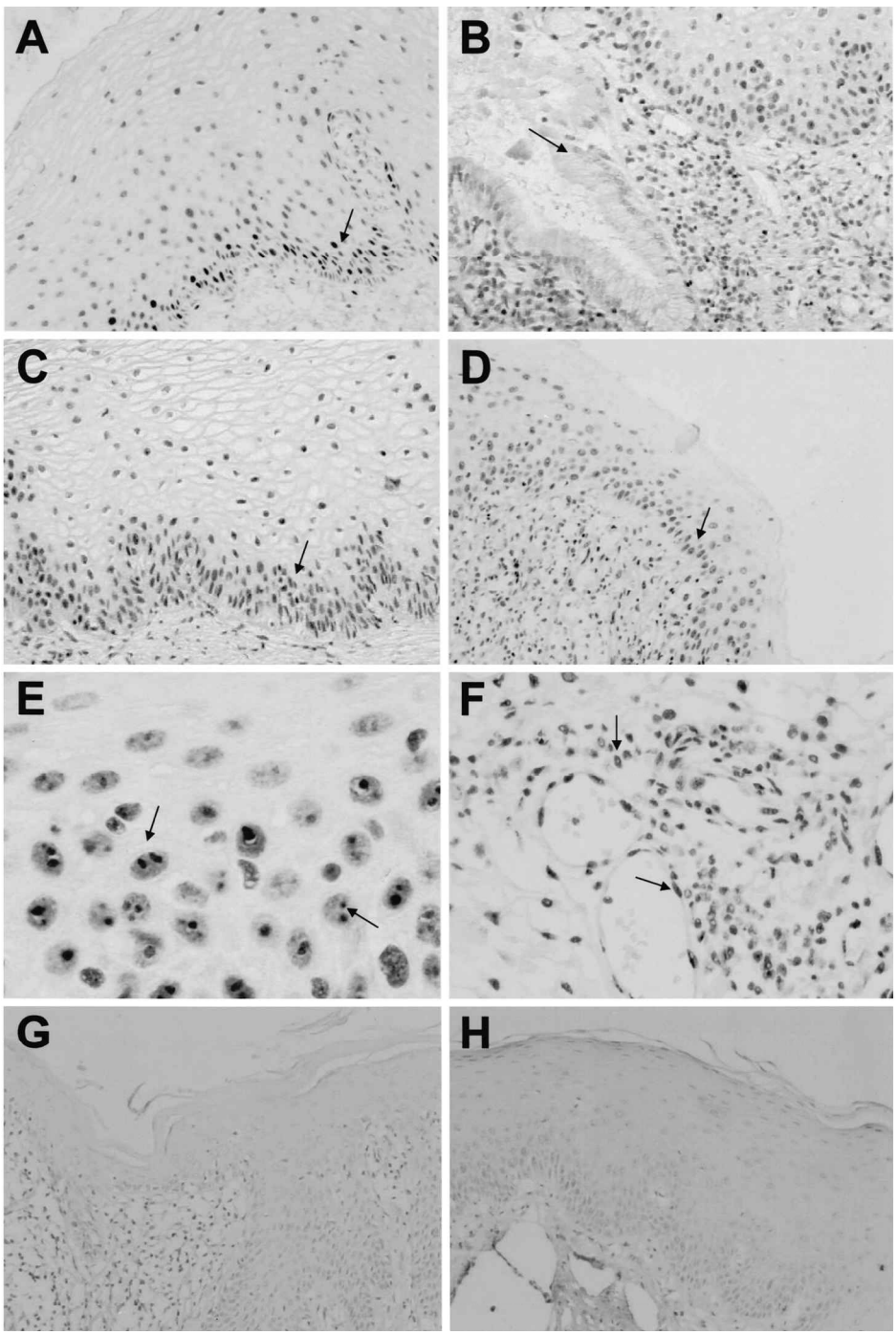

FIG. 2. Immunohistochemical detection of IFI16 protein in normal human tissues. (**A**) Exocervix. (**B**) Exo-endocervix junction. The epithelial cells lining the exocervix are positive, but those lining the endocervix are not (arrow). Notice that the inflammatory cells strongly expressed IFI16. Nuclei of cells not expressing IFI16 are stained blue by counterstain. (**C**) Vocal cord epithelium. (**D**) Skin. (**E**) High-power field of IFI16-positive cells showing nucleolar staining (arrow). (**F**) Strong IFI16 immunoreactivity in stromal reactive (upper arrow) and endothelial cells (lower arrow). (**G**) Skin stained with preimmune serum. (**H**) Vocal cord epithelium stained with preimmune serum. Arrows in **A, C, D** indicate IFI16-positive cells. **A, B, C, D, G, H,** ×10. **E,** ×40. **F,** ×20.

tein Assay (Bio-Rad Laboratories, Hercules, CA). Total cell extracts were separated on SDS-8.5% PAGE and transferred onto PVDF membrane (Amersham, Arlington Heights, IL). Membranes were blocked in blocking solution (10 mM Tris-HCl, pH 7.5, 0.1 M NaCl, 0.1% Tween 20, 5% [w/v] nonfat dry milk) and incubated with affinity-purified polyclonal antibody (diluted 1:5000) generated in rabbit or anti-IFI16 monoclonal antibodies (mAb) kindly provided by J. Trapani. The appropriate horseradish peroxidase (HRP) conjugate was used as second antibody at a dilution of 1:4000 and detected by ECL (Amersham). Monoclonal anti-actin antibodies (Boehringer, Mannheim, Germany) were used as internal control.

Immunohistochemistry

Archival, formalin-fixed, paraffin wax-embedded normal tissues were obtained from the Department of Surgical Pathology, San Giovanni Hospital (Turin, Italy). Sections 4 μm thick on silane-coated glass slides were dewaxed by passage through xylene, and the endogenous peroxidase activity was blocked with 0.3% H_2O_2/methanol. For IFI16 antigen retrieval, the slides were placed in Coplin jars filled with a 10 mM trisodium citrate solution at pH 6.0 and heated for 26 min at 750 W and then for 15 min at 300 W in a conventional pressure cooker cycle in a microwave oven. Slides were incubated in blocking solution (5% donor serum plus 0.2% Triton-X100 in PBS) to prevent nonspecific binding and then with an optimal dilution of the primary antibody (1:5000 for anti-IFI16). After washing in PBS, slides were incubated with the appropriate biotinylated secondary antibody, followed by streptavidin-HRP complex. Immunostaining was by incubation of the slides in diaminobenzidine solution (DAB) (Immunotech, Milan, Italy). Finally, slides were counterstained with hematoxylin for 30 sec, dehydrated, and mounted with mounting medium (Eukitt) (Bioptica, Marseille, France). In all experiments, a routine control was included in which the primary antibody was omitted. Substitution of the antibody with preimmune serum was the internal control.

RESULTS

Polyclonal monospecific anti-IFI16 antibodies specifically recognize three isoforms of IFI16 gene products

IFI16 is constitutively expressed in lymphoid cell lines but is inducible with IFN-α or IFN-γ and chemical agents eliciting differentiation in myeloid cell lines, such as HL-60 and U-937. To see whether the anti-IFI16 antibodies raised in rabbits were able to detect IFI16, we performed a Western blotting analysis with total cell extracts from both untreated and IFN-γ-treated cell lines. We chose a 24-h IFN treatment because time course experiments have shown that IFI16 induction peaks at 12 h and is then maintained at maximal levels. As shown in Figure 1, the affinity-purified antibodies recognized, in total extracts from IFN-treated cells, three closely migrating bands with apparent molecular weights ranging from 85 to 95 kDa. The central band was the most prominent. The three isoforms were identified in IFN-γ-treated immature myeloid cell lines (HL-60 and U-937) and in the IFN-γ-treated epithelial cell line HaCat. This cluster of proteins represents the typical IFI16 pattern in SDS-PAGE analysis reported by Dawson and Trapani[20] with a monoclonal anti-IFI16 antibody (Fig. 1, lane mAb). The three isoforms (A, B, and C) are the result of alternative mRNA splicing and differ in the size of their hinge domain, which can consist of one, two, or three multiples of a 56-amino acid motif encoded by a single copy of exon 6 or two reiterated copies of exon 7.[10] As expected, the preimmune serum did not recognize any band.

Immunohistochemical detection of IFI16 protein in normal tissues

Immunohistochemical methods are used for rapid and sensitive screening of tissues to establish the physiologic expression pattern of a protein in tissues. Therefore, this technique was set up for the detection of the IFN-inducible protein IFI16 using the polyclonal antibodies described. Preliminary experiments determined the optimal antigen retrieval technique and dilution. The best staining was achieved with the citric acid buffer and a combination of heat and pressure as described in Materials and Methods. Since the antibodies generated the same staining pattern with both fresh frozen and formalin-fixed, paraffin-embedded tissue sections, the latter was chosen for practical reasons.

Formalin-fixed, paraffin-embedded sections of normal adult tissues obtained from surgical specimens were stained with anti-IFI16 polyclonal antibodies. As a negative control, the preimmune serum was used, and no staining was obtained (Fig. 2G,H). The IFI16 intracellular staining pattern was similar in different organs. As shown in Figure 2E, the immunostaining is mainly nucleolar, in accordance with immunofluorescence studies earlier performed using a mAb detecting IFI16 and, therefore, dependent on the presence of nucleoli.[22] Thus, only transcriptionally active cells, which transcribe ribosomal DNA in the nucleoli, express IFI16. A different expression between connective tissue and hematologic cells was seen in mesenchymal tissues (Table 1). IFI16 was almost absent in connective tissue. Myocytes did not express it, and few fibroblasts were stained. Interestingly, reactive fibroblasts and myofibroblasts from inflammatory tissues strongly expressed IFI16. In accordance with previous reports, prominent expression was seen in monocytes and lymphocytes, particularly in germinal centers. In contrast, only some resident macrophages expressed the protein, and granulocytes were negative.[19–21] All vascular endothelial cells, from both hematic and lymphatic vessels, strongly expressed IFI16 (Fig. 2F).

Wide variations in IFI16 distribution in epithelial cells were seen, with different patterns in different organs and in lining and glandular epithelia. Epithelial cells of the skin, digestive tract, respiratory tract, urinary tract, reproductive tract, and internal parenchymatous organs were investigated. Some representative results (Fig. 2) can be best described in relation to the layers of the epidermis, which consist of epithelial cells with different degrees of differentiation. The basal layer of the epidermis contains proliferating cells and is laid on the basement membrane. As epidermal cells enter the suprabasal prickle layer, they begin to differentiate and move toward the outer granular and keratinized layers and are eventually sloughed off. IFI16 expression was detected in parabasal cells in the proliferating compartment, gradually decreasing in the suprabasal,

more differentiated compartment. Representative photographs showing the range of staining patterns are shown in Figure 2D.

Regions of the gastrointestinal tract, such as the oral cavity, esophagus, and anal canal, that are lined with stratified squamous epithelium displayed strong IFI16 staining in the parabasal compartment. Weak and sporadic positivity was observed in more differentiating layers of the squamous epithelial lining of these regions (Fig. 2C). The epithelium of the gastrointestinal tract presents an abrupt morphologic change at the esophagogastric junction, as did IFI16 expression. Its expression was quite robust in the esophagus and disappeared in the surface epithelium of the stomach. Both glandular and foveolar gastric epithelia were completely negative. Mucosal epithelium of small and large bowel did not express the protein. Interestingly, a weak positive stain was observed in some glandular cells of the upper digestive system, salivary glands, and submucosal esophageal glands but was absent in the glandular epithelium of the lower digestive regions. Ductal lining of salivary glands expressed IFI16 in basal layer cells but not in the luminal layer.

In the respiratory system, the vocal cord mucosa, which is lined by a thick stratified squamous epithelium, expressed IFI16 in the parabasal compartment. The ciliated pseudostratified epithelium of the upper respiratory tract also expressed IFI16, but

Table 1. Immunohistochemical Detection of IFI16 Protein in Human Normal Tissues

	Epithelial cells		*Nonepithelial cells*	
Tissue	*Cell type*	*Expression*	*Cell type*	*Expression*
Skin	Epidermis	+++	Fibroblasts	±
Oral mucosa/ oropharynx	Epithelial lining	+++	Myocytes	−
	Glandular epithelium	+		
	Ductal epithelium	++	Lymphocytes	+++
Larynx	Vocal cord epithelium	+++	Monocytes	+++
	Respiratory epithelium	++		
Lung	Respiratory epithelium	−	Macrophages	±
	Glandular epithelium	+	Granulocytes	−
Esophagus	Lining epithelium	+++	Endothelial cells	+++
	Submucosal glands	+		
Stomach	Foveolar epithelium	−		
	Glandular epithelium	−		
Bowel	Small bowel	−		
	Large bowel	−		
Kidney	Tubular epithelium	+		
	Urothelium	−		
Bladder	Urothelium	−		
Testis	Leydig cells	−		
Exocervix	Lining epithelium	+++		
Endocervix	Lining epithelium	−		
	Glandular epithelium	−		
Endometrium	Glandular epithelium	−		
Liver	Hepatocytes	−		
	Ductal epithelium	−		
Pancreas	Glandular epithelium	−		
	Ductal epithelium	+		
	Island epithelium	−		
Thyroid	Follicular epithelium	−		

+, few positive cells; ++, moderate positive cells; +++, many positive cells; ±, few or no positive cells, −, no positive cells.

only a few cells of the lower respiratory tract of the trachea, bronchi, and bronchioli were positive. Glandular serous and mucous appendages of these regions were negative, and only the basal layer of the ductal epithelium was positive, like the ductal epithelium of salivary glands.

In the urinary tract, the IFI16 distribution displayed regional differences. In the kidney, tubular and ductal epithelial cells were positive, whereas the urothelium, which lines the lower regions of the excretory system, was almost negative. The regional distribution in the kidney reflected the different embryologic derivations of the parenchymal and excretory compartment.

The vaginal surface and the epithelium of the outer surface of the portio vaginalis (exocervix), which is lined with stratified squamous epithelium, expressed IFI16 in the parabasal proliferating compartment of the mucosa (Fig. 2A). In contrast, the glandular mucosa of the endocervix, formed by a layer of columnar mucus-secreting cells, was not stained (Fig. 2B). Squamous metaplasia of the cervical transitional zones and tubal metaplasia strongly expressed IFI16. The lining and glandular mucosa of the endometrium did not express IFI16.

Germinal cells in the testis, such as Leydig cells did not show protein expression, whereas some Sertoli cells were weakly positive. Hepatocytes and bile duct cells in the liver, pancreatic acinic and ductal cells, adrenocortical glands and medullary cells, thyroid follicular cells, and islets of Langerhans and other APUD system endocrine cells did not express IFI16.

DISCUSSION

Information about *in vivo* expression of IFI16 is essential for interpretation of the results obtained from *in vitro* studies and elucidation of its physiologic role. In this study, we evaluated the distribution of IFI16 immunohistochemically in a wide variety of normal human tissues. An affinity-purified rabbit polyclonal antibody was used, whose specific affinity toward IFI16 proteins was evaluated by Western blotting.

IFI16 was originally isolated by screening a λgt11 expression library constructed using RNA from interleukin-2 (IL-2) augmented mixed lymphocyte culture cells with xenoantisera raised against subcellular lytic granules from the same cells. The study was undertaken to identify novel proteins expressed in human lymphoid cells, particularly cytotoxic T lymphocytes (CTL) and natural killer (NK) cells.[20] Since its original cloning, this gene has been considered specific for hematopoietic cells.[21] Its expression has been investigated mostly in this compartment, and its normal pattern is still uncertain. Our evaluation of the immunohistochemical localization of IFI16 in human organs demonstrates that its expression is highly selective. In accordance with the literature, strong IFI16 expression was observed in lymphocytes and monocytes. In contrast, only some resident macrophages were stained, and granulocytes were negative. In normal adult human tissues, IFI16 is expressed in a highly restricted pattern in selected cells within certain organs. It is located predominantly in the nucleus, with prominent nucleolar staining, as in previous reports, showing that IFI16 is mainly nucleolar.[22] Prominent IFI16 expression is seen in stratified squamous epithelia and is particularly intense in parabasal cells in the proliferating compartments and gradually decreased in the suprabasal, more differentiated compartment. In the cervix, only the squamous epithelium of the exocervix was positive, and the glandular mucosa of the endocervix was negative. Interestingly, both squamous metaplasia of the cervical transitional zones and tubal metaplasia strongly expressed IFI16. This finding, together with the observation that reactive fibroblasts and inflammatory cells in the submucosa are strongly IFI16 positive, indicate that its expression may be associated with reactive cells and its expression be upregulated in response to deregulated proliferation.

The constitutive expression and wider distribution of IFI16, not restricted to the hematopoietic compartment, in human normal tissues support the possibility of an important regulatory role that can be further modulated by other stimuli, such as IFN.

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